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Anti-RNA Antibodies

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SPÉCIFICITÉ DES ANTICORPS ANTI-RNA
SPECIFICITY OF ANTI-RNA ANTIBODIES

by

E. Barbu, G. Quash, and J.-P. Dandeu

The presence of anti-RNA antibodies in the sera of animals immunized with ribosomes has been observed as a result of the specific precipitate which is formed when RNA is added to these sera (Barbu and Panijel, 3,4).

The presence of anti-RNA antibodies in human sera and in nonimmunized animals has been demonstrated using polyinosinic acid "poly A" for precipitation (Barbu and Dandeu, 2).

Presently, the problem of the specificity of these antibodies is dealt with. With this regards, in a previous study the heterogeneity of these antibodies was noted (Barbu and Dandeu, 1). Results similar to these have been obtained by Panijel (20).

METHODS AND MATERIALS

(1) Antisera

Antiserum containing anti-RNA antibodies has been obtained by intravenous inoculation of horse no. 482 with 1.5 gm of ribosomes prepared from Proteus vulgaris given in doses of 10 to 30 mg.

Crude horse antisera (anti-tetanus, anti-diphtheria, and anti-human) were obtained from the Pasteur Institute. Anti-tetanus serum and purified tetanus toxin were obtained from Mr. Turpin.

Anti-human-γ-globulin sera were received from Mr. Sandor.

Horse anti-rabbit-γ-globulin sera were prepared by immunizing rabbits with the specific precipitates formed by adding polyadenylic acid (poly A) or RNA to horse serum no. 482. These experiments were conducted with the object of obtaining directly anti-"poly A" or anti-RNA antibodies but were not carried out beyond two months so that the rabbits obtained a total of 15 mg of "poly A" or RNA.

Human sera were provided by the Transfusion Center of the Pasteur Institute.

Chicken sera were received from Mr. Atanasiu.

(2) Isolation and Fractionation of Anti-RNA Antibodies

(A)- starting with horse antiribosomal serum no. 482-- to 100 ml of serum is added 1.0 ml of a "poly A" solution containing 5 mg per ml. The mixture is maintained at 4°C for 4 hr and then centrifuged; the specific precipitate is washed twice with saline solution (0.1 M NaCl + 0.01 M magnesium acetate + 0.005 M tris, pH 7.4). Next, to the precipitate are added 20 ml of 1.0 M $MgCl_2$; This mixture is kept at 4°C and is lightly stirred from time to time. After 24 hr, the "poly A" precipitate is removed by centrifugation. The antibody, about 35 mg, stays in solution and is dialyzed against a saline solution "φ" (0.14 M NaCl + 0.005 M tris, pH 7.4). This antibody fraction is designated at fraction "a".

The serum supernatant obtained after precipitation with "poly A" is treated a second time with 5 mg of"poly A". To 100 ml of the serum, thus extracted with "poly A", are added 5 ml of a "poly I" solution containing 10 mg per ml. The precipitate that forms is treated as previously described and yielded about 80 mg of antibody. This fraction was designated fraction "b".

When "poly I" is added to antiserum not previously extracted with "poly A", a precipitate, designated as fraction "I", is obtained which contains both the antibodies of fraction "a" and those of fraction "b". This mixture of antibodies obtained after separation with "poly I" is unstable at 4°C and forms a precipitate at that temperature which can be redissolved at 37°C.

In the same way, the anti-RNA antibodies are isolated from horse serum using ribosomal RNA; the antibodies thus obtained are unstable at 4°C and the total yield of antibody is smaller than that obtained when the serum is extracted first with "poly A" and then with "poly I".

The fact that the antibodies in fractions "a" and "b" are more stable when separated, even after several months at -15°C, led us to suppose that the instability is probably due to the formation of complexes between the antibodies of fraction "a" and those of fraction "b".

(B)- starting with non-immune human or animal sera--- in this case, we were able to obtain a precipitate with "poly I". In order to isolate the natural antibodies designated "γI", 2 to 5 mg of "poly I" were added to 10 ml of serum. The precipitate was treated as before and yielded 1 to 2 mg of "γI" per 10 ml of serum.

It was observed that the polynucleotide or RNA used for precipitation can be recovered after separation of the globulins in the presence of $MgCl_2$.

In order to economize of "poly I", the practice of initial concentration of the "γI" was considered. In this case, it has been observed during dialysis of the antisera against distilled water, that almost all of the "γI" is recovered in the euglobulin fraction. This can be redissolved in the "φ" solution, then the "γI" is precipitated with "poly I"

from this euglobulin solution.

(3) Ribonucleic Acid

This was prepared by the phenol method (Schuster et al., 25) in the presence of bentonite according to the method of Fraenkel-Conrat et al. (10).

(4) Polyribonucleotides

Part was donated by Mme. Grunberg Manago while the remainder was obtained from Miles Chemical Co., U.S.A.

(5) Tobacco Mosaic Virus (TMV)

Obtained from Mr. Cornuet.

(6) Specific Precipitin Reactions and Amounts of Precipitate

These were carried out using the methods described in previous studies (Barbu et al., 5).

(7) Immunoelectrophoresis Experiments

These were carried out using the techniques of Grabar et al. (11); those of gel diffusion using the techniques described by Ouchterlony (18).

RESULTS

A.
Properties of Proteins precipitated by RNA or Polyribonucleotides

These proteins were studied using paper electrophoresis, immunoelectrophoresis, gel diffusion, and chromatography on Sephadex G-200.

(1) Paper elcctrophoresis was carried out in veronal buffer, pH 8.2. The

curves in Figure 1 show the absorption of the protein bands separated by paper electrophoresis and colored with the amido-Schwarz dye. It can be seen that the two fractions "a" and "b", separated from serum no. 482 as described, migrated towards the negative pole; but fraction "a" behaved as if its isoelectic point was at a higher pH than that of fraction "b". Fraction "I" was more spread out; it included both the antibodies of fraction "a" and "b".

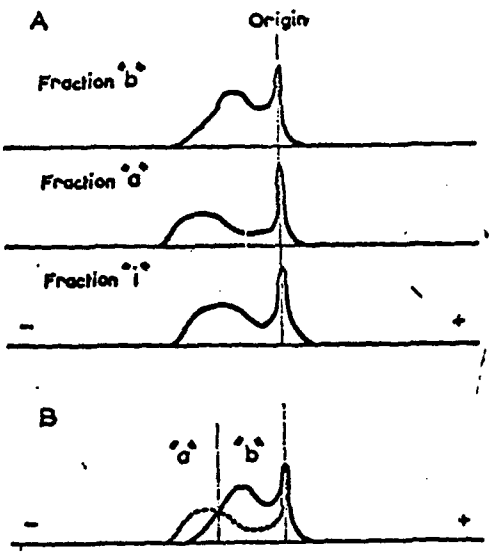

Fig. 1- Absorption of protein bands separated by electrophoresis. A, fractions "a", "b", "I"; B, superposition of tracings of fractions "a" and "b".

(2) Immunoelectrophoresis was carried out in veronal buffer, pH 8.2. It is shown in Figures 2 and 3B that the antibodies precipitated from horse serum no. 482, fraction "I", as well as "γI" from human serum, behave as γ-globulins. The rabbit serum against horse serum no. 482

was a very good anti-horse-γ-globulin serum has can be seen from its reaction with whole horse serum.

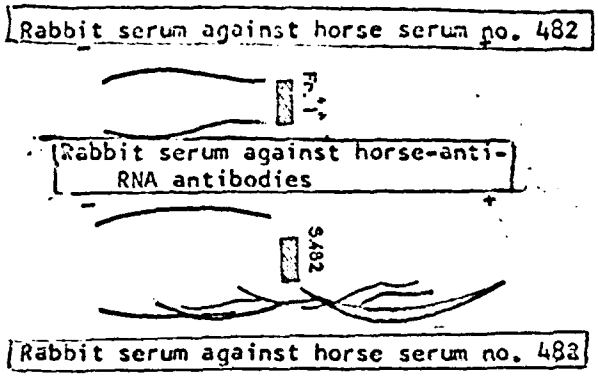

Fig. 2- Immunoelectrophoresis of antibody fraction "I" and horse serum no. 482 from which this fraction was isolated.

(3) Gel diffusion studies were carried out with fraction "γI" from human serum. The results obtained are shown in Figure 3. The "γI" gives a single precipitation band which shows a continuity with that of a purified human γ-globulin fraction in the presence of antiserum against whole human serum.

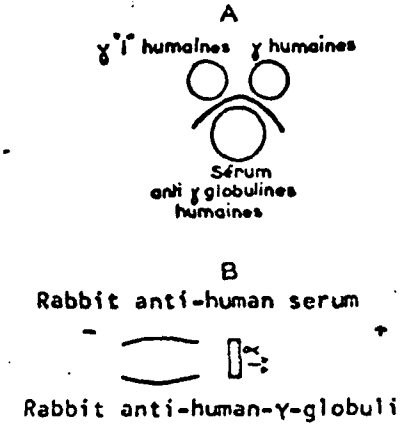

Fig. 3- A: comparison of human fraction "γI" and purified human γ-globulin using gel diffusion and anti-human-γ-globulin serum; B: Immuno-electrophoresis of the human "γI" fraction.

(4) Chromatography on Sephadex G-200-- It has been shown that almost all of fraction "b" is retained on the column as long as the control preparation of γ-globulin. This, and the fact that the reactions of "poly I" with fraction "b" as well as with "γI", are not decreased to any extent by treatment with mercaptoethanol according to Deutch and Morton (8) led us to conclude that these fractions of γ-globulins contain only a very small proportion of macroglobulin.

(5) In conclusion, the proteins precipitated with RNA or polynucleotides from sera of animals immunized (1) against ribosomes or from human sera are γ-globulins, electrophoretic fraction γ_2 in the case of human sera.

B.

Specific Precipitation of anti-RNA Antibodies

The precipitin reactions were carried out in the following manner: to 1.0 ml of serum is added 0.1 ml of a solution of polynucleotide or RNA in solution " ". The mixture is held for 4 hr at 4°C and then centrifuged. The precipitate is washed twice with 2.5 ml of the wash solution and the RNA and proteins are determined.

This has been carried out on the sera of animals immunized with ribosomes, on antibody fractions previously isolated from these sera as well as from human sera and from the sera on nonimmunized animals.

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(1) The γ-globulin nature of the anti-RNA antibodies of rabbit anti-ribosomal sera has been previously proven by immunoelectrophoresis (Barbu and Panijel, 2). In addition, these antibodies fix complement in the presence of RNA (Stahl and Barbu, 27) which serves to emphasize the immunological character of these reactions.

The results obtained are summarized in Table I through III. In the case of horse serum no. 482, it was determined (table I) that the quantities of antibody precipitated by "poly I" are more important than those precipitated by ascites RNA, but the latter precipitated twice as much antibody as "poly A".

The antibody/antigen ratios are clearly much higher in the case of RNA than with the polynucleotides.

When the serum has been extracted with "poly A", ascites RNA brings down only a very small amount of antibody in the precipitate; in contrast, "poly U" (polyuridylic acid) and "poly I" elicit the precipitation of important quantities of antibody.

Extraction with "poly A" does not change the antibody/antigen ratio in the case of precipitation with "poly I"; in contrast, the ratio is considerably decreased when RNA is used for precipitation.

Table II presents the results obtained with fractions "a" and "b" from horse serum no. 482.

It was established that RNA from liver or ascites, as well as "poly A" or "poly U" carry down in the specific precipitate 50 to 60 % of fraction "b" while "poly C" precipitates only 17 %.

The antibodies of fraction "b", isolated with "poly I" and which do not precipitate with "poly A", do precipitate in the presence of "poly U" to the extent of 30 %. They yield very scanty precipitates with liver RNA (in the order of 4 %), but with TMV RNA, the precipitation is increased to 16 %.

Fraction "b" precipitates with "poly I" even after extraction with liver RNA.

Table I. - Constitution of specific precipitates from 1 ml of antiserum.

antisera	antigens	µg antigen added per 1 ml serum	µg antibody in specific precipitate	percentage antigen precipitated	µg antibody / µg antigen
Horse Serum no. 482	Ascites RNA	13.5	440	85	38
		29.5	692	70	34
		52.0	840	62	26
		103.5	856	38	22
		207.0	816	24	18
	poly A	20	310	90	17
		40	360	89	15
		81	390	60	8
		163	410	31	8
		326	400	16	7.5
	Poly I	90	630	54	13.3
		180	980	41.5	13.5
		360	1,670	39.5	12.1
		720	2,440	40	8.8
		1,380	3,100	31.5	7
		2,750	3,180	24.2	4
		5,500	3,050	22.5	2.7
		11,000	2,240	13.7	1.4
Horse serum #482 extracted with poly A	Ascites RNA	12	72	100	6
		25	88	100	3.3
		49	78	69	2.5
		97	23	13	2
		390	5	0.5	3
	Poly U	17	85.8	94	5.3
		32.5	169	66	8
		70	363	61	9
		140	628	52	9
		280	1,053	40	9.5
		560	1,352	37	9
	Poly I	30	24	15	5
		62	109	17	10
		125	344	18	13
		250	848	20	13
		500	1,600	25	13

Table II- Compostion of specific precipitates from 1 ml of antibody solutions

Antibodies	Antigens	µg antigen added	µg antibody precipitated	percentage antigen precipitated	µg antibody / µg antigen
		14	287	100	20
		28	336	100	12
	Ascites	88	480	88	6
	RNA	176	448	65	4
		354	240	25	3
		34	384	83.5	13.5
		70	431	57	10.7
	Liver	140	480	41.8	8.2
	RNA	280	480	24.2	5
		568	464	24.5	3.3
		14	336	100	20
		28	351	100	12
	Poly A	109	384	48	7
fraction		218	400	25	7
"a"		436	431	12	8
860 µg		18	152	44	19
per ml		36	224	27	22
		72	336	27	17
	Poly U	146	464	21	15
		292	480	14	9
		582	496	9.9	8.7
		16	81	46.5	10
		32	85	34.5	8
	Poly C	66	111	23.2	7.2
		133	121	11.6	7.5
		266	137	7.9	7
		50	50	12	8.3
		100	64	10	6.4
	Poly U	200	68	13	2.7
		400	92	--	--
		800	70	12.8	0.6
fraction		1,600	68	14	0.3
"b"		16	32	17	11.8
310 µg	TMV	32	33	10.9	9.4
per ml	RNA	64	34	8.4	6.2
		128	48	6.4	5.2
		25	7	--	--
fraction		50	9	--	--
"b"	Liver	100	11	--	--
288 µg	RNA	200	12.5	--	--
per ml		400	11.5	--	--
fraction		15.5	48	53	6
"b"		31	54	30	5.3
extracted	Poly I	62	74	27.4	4.6
with liver		124	88	27	2.3
520 µg per ml		248	118	14	--

Table III- Micrograms of protein precipitated from 1 ml of serum using "Poly I"

Nature of Sera	µg "Poly I" added per ml serum				
	62	125	250	500	1000
Non-immune rat serum:					
1	--	20	--	--	--
2	--	32	--	--	--
Non-immune sheep serum:					
1	64	55	88	--	--
2	24	40	86	--	--
Non-immune rabbit serum:					
1	15	15	34	--	--
2	36	26	34	---	--
Horse serum, non-immunized	--	138	150	218	265
" " anti-tetanus toxin	--	16	30	54	58
" " anti-diphtheria toxin	--	8	10	14	22
Serum from 20- 19 day old chick embryos	--	--	117	143	130
Serum from 4 chicks of age:					
8 days	78	95	108	127	160
15 days	102	119	133	142	160
30 days	166	186	213	256	307

These results, as well as those in Table I, verify both the heterogeneity of anti-RNA antibodies and the differences in behavior of the various RNA's and polyribonucleotides.

In Table III are presented the results obtained with nonimunized and immunized animal sera using testing antigens other than ribosomes. It can be seen that "poly I" elicits precipitation of "γI" protein in all of the sera, but this precipitation is very much less than those obtained in the case of animals immunized with ribosomes (Table I and reference 2). In the case of the sera of horses immunized with tetanus toxin or with diphtheria toxin, the amount of "γI" is significantly less than that recovered from three nonimmunized horses.

The quantity of protein precipitated with "poly I" increases with the age of the animal as can be seen in the case of the chick.

With regards to human sera, we previously reported the existence of differences in the amount of "γI" in sera of normal subjects and in those with pathological conditions (Barbu and Dandeu, 2).

A fraction of "γI" has been isolated from the serum of a blood donor which, at a level of 300 µg per ml, does not precipitate in the presence of liver ribosomal RNA, but gives a precipitate containing 22µg of antibody and 7 µg of RNA with TMV RNA.

C.

Inhibition of Precipitation of Anti-RNA Antibodies

A systematic study of the inhibition of specific precipitation of these antibodies has not been undertaken. The studies have been limited to the precipitation of fraction "b" and "γI" by "poly I" in the presence of RNA, DNA, "Poly A", or in the presence of 0.4 M NaCl.

It has been observed (Table IV) that liver RNA, which does not precipitate with "γI", inhibits precipitation with "poly I". There is then in any case specific interactions between this RNA and γI. The same is true in the case of the inhibition by "Poly A" of the precipitation of fraction "b" by "Poly I".

DNA, which does not precipitate with these fractions, does not inhibit in any way precipitation of "poly I" with fraction "I" of horse serum no. 482 or with the "γI" fraction of human serum.

The precipitation of antibodies in horse serum no. 482 by RNA or by "Poly I" is strongly inhibited in the presence of 0.4 M NaCl (Table V).

This inhibition is more important in the case of "poly I" than with RNA, which implys that it affects the antibodies of fraction "b" more than those of fraction "a". Furthermore, the quantity of antibody precipitated in the presence of 0.4 M NaCl by RNA or "poly A" is in the same order as that precipitated in the presence of "poly A" (Table I).

DISCUSSION

The specificity of anti-protein antibodies is such that these antibodies react only with the protein used for immunization or with protein antigens which possess antigenic sites similar to those of the protein used for immunization.

In the case of anti-RNA antibody, the situation is different from that of anti-protein antibodies in a number of ways. For example, no evidence has been obtained to show that anti-RNA antibodies react only with the RNA used for immunization; actually, an anti-bacterial ribosomal serum can freed completely of its anti-RNA antibodies by absorption with

synthetic polynucleotides such as "poly I"; nevertheless, all of the polynucleotides or almost all of the RNA do not succeed in extracting an anti-ribosomal serum.

These facts emphasize the heterogeneity of anti-RNA antibodies (Barbu and Dandeu, 1) and the important contribution, in these reactions, of the determinant common to all RNA's and polyribonucleotides, the polyribose phosphate. This does not exclude the possibility of contributions from the RNA bases to the antibody reaction. They are able, for example, to affect the production of more stable RNA-antibody complexes[2], but in order for antibodies to attach to RNA, it is not necessary that the site of fixation have base determinants linked in a certain order. It is, therefore, very difficult to conceive of a separation of these antibodies based on their ability to recognize the order of nucleotide arrangement; nevertheless, this is not eliminated a priori; actually, in the case of enzymes transferring amino acids and with basic proteins (Quash et al, 22) as "repressors" (Monad et al., 17), the possibility of recognition by the proteins of the order of nucleotide arrangement has to be admitted.

---

[2] It has been observed that "poly I" and "poly U", which give precipitation reactions with all of the antibody fractions thus far isolated, can be characterized by the absence of NH_2- groups. The same is true in the case of polydeoxythymidylic acid which, according to Levine (16), gives a very strong reaction with anti-DNA antibodies.

We have, therefore, introduced another hypothesis to explain both the heterogeneity of anti-RNA antibodies and the differences in behavior or RNA and various polyribonucleotides during the precipitation reaction.

The ribose phosphate must certainly constitute a part of the antigenic sites of RNA, because DNA will not react with anti-RNA antibody; furthermore, the reverse is true: anti-DNA antibodies will not react with RNA (Seligmann, 26; Barbu et al., 6; Levine, 16). We have been able to compare the behavior of these to ribonucleases and deoxyribonucleases with regards to their respective substrates.

If the ribose phosphate grouping is involved in the reaction, one ought to ask how it affects the interaction of antibody with RNA after the former has been fixed to the RNA. In this regards, Kabat (12) has considered the existence of different antibodies according to the length of the dextran chain which was recognized (3 to 7 units of glucose) in t the case of anti-dextran antibody.

It is not the number of units (ribose phosphate or glucose) that the antibody recognizes but rather the presence on the antibody surface corresponding to each unit.

Differences in secondary structure have been recognized on account of different catagories of RNA and polyribonucleotides. These differences in secondary structure correspond more or less to the arrangement of their units adapted to the arrangement of corresponding sites on the antibody.

These results have been used to explain in this way both the heterogeneity of anti-RNA antibodies and the differences in behavior of RNA and polyribonucleotides with regards to the precipitation reaction.

With resgrds to the heterogeneity of anti-RNA antibodies present in horse anti-Proteus vulgaris ribosomal serum, presently two large large fractions have been characterized: fraction "a" whose antibodies are precipitated with "poly A", and fraction "b" whose antibodies are precipitated with "poly U" or "Poly I" after extraction of the serum with "poly A"[3].

These two fractions can be distinquished not only by their immunological specificities, but also by their physico-chemical properties. As shown in Figure 1, fraction "a" is located after electrophoresis closer to the negative pole than fraction "b". This basic characteristic has been confirmed by the preferential adsorption to bentonite after elimination of serum ribonuclease.

The "γI" fraction precipitated with "poly I" from human sera and from the sera of two non-immunized animals behaves similarly to the antibodies in fraction "b"; it precipitates weakly with ribosomal RNA.

All of the preparations of "γI" precipitate with "poly I", "poly U", or with TMV RNA, but give only a weak reaction with ribosomal RNA (in the order of 5 to 20 % that obtained with "poly I").

The antibodies of fraction "b" or of "γI", which do not precipitate with ribosomal RNA, exhibit in any case interactions with ribosomal RNA or with "poly A" as suggested by theinhibition by these of the precipitation of "poly I" with these antibodies (Table IV).

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[3] Certain of our gel-diffusion experiments (Barbu and Dandeu, 1) as well as others after extraction of fractions "a" and "b" with different polyribonucleotides suggest the possibility of distinguishing subfractions, but this has not been studied thoroughly.

The anti-polyribonucleic specificity of "γ" has been confirmed by the fact that, in the case of hyperimmunized horse sera for tetanus or diphtheria toxins, one has not been able to find anti-tetanus or anti-diphtheria antibodies in the "γI" fraction. The contrary would have represented a very serious arguement against the specificity of precipitation with "poly I". An arguement in favor of this specificity is the increase in amount of precipitation resulting from the immunization of animals (rabbit, horse, goat) with bacterial ribosomes (Barbu and Dandeu, 2).

In addition, it has been shown that precipitation of the antibodies of fraction "a" is inhibited less in the presence of 0.4 M NaCl than are those of fraction "b". Everything, therefore, seems to indicate stronger interactions with polyribonucleotides in the case of fraction "a" than with fraction "b".

The differences in behavior of the various RNA's and polyribonucleotides in the presence of fraction "a", fraction "b", or with antiserum containing both, needs to be interpreted if one is to take account of their secondary structure.

It follows from these results that the polyribonucleotides possess a paired chain structure since "poly A" (Rich et al, 24), "poly C" (Langridge and Rich, 15), or transfer RNA (Brown and Zubay, 7) give a precipitation reaction with fraction "a" and only a weak reaction with fraction "b"; while "poly U" and TMV RNA, which exhibits a unique easily distorted chain, are able to react with the two fractions.

Ribosomal RNA likewise reacts with the two fractions, nevertheless, the reactions with fraction "b" or with "γI" are weaker than those with TMV RNA. It has been shown that the guanine content of this RNA is significantly less than that of ribosomal or transfer RNA (Doty et al, 9).

The differences in precipitation demonstrated in the case of whole antisera between soluble RNA and ribosomal RNA can be interpreted in the same way (Panijel et al., 21; Panijel, 19; Lacour et al., 13, 14).

The case of "poly I", which has been described as having a triple chain structure, is more difficult to explain (Rich, 23). Nevertheless, it appears to be a good "messenger" in in vitro amino acid incorporation experiments.

It has been shown in preliminary experiments that precipitation of horse serum no. 482 with "poly A" is sensitive to temperature. The specific precipitate forms at 37°C but goes back into solution at 4°C. This suggests a modification of secondary structure of "poly A" as a function of temperature. The antibodies themselves can't be responsible for this since they have already been demonstrate to be as stable at 37°C as they are at 4°C. An analogous temperature effect has been shown in the case of the inhibition by "poly A" of the precipitation of "poly I" with fraction "b" (Table V).

Experiments are presently in progress to study the relationships between secondary RNA or polyribonucleotide structures and their precipitation with various antibody fractions.

At present, if one assumes that the structure of TMV RNA is similar to that of certain RNA messengers, but contains less guanine than ribosomal or transfer RNA (Doty et al., 9), one can expect to preferentially precipitate them with "γI" or with fraction "b" from horse serum.

SUMMARY

The anti-RNA antibodies present in the sera of animals immunized with ribonucleoproteins (ribosomes) have been characterized as γ-globulins. This character "γ-globulinic" is also exhibited by the proteins "γI" precipitated by "Poly I" from human sera and from the sera of nonimmunized animals.

The serum titer of these proteins "γI" increases with the age of the animal (chicken) and even much greater increases are found when animals are immunized with ribosomes. On the other hand, in sera containing antibodies to other antigens, the "γI" titer does not increase, but sometimes even diminishes as immunization is continued.

From an anti-ribosomal horse serum, two broad categories of antibodies to RNA, "a" and "b", exhibiting physico-chemical and immunological differences have been isolated.

These fractions "a" and "b" interact with varying intensity with all of the polyribonucleotides and RNA used, but differ in their respective abilities of forming soluble or precipitating complexes with some of them.

Their specificity is such, that they react uniquely with polyribonucleotides and more specifically with the polyribose phosphate groups. It is on the availability of the polyribose phosphate group (itself dependent on the secondary structure of the polyribonucleotides or RNA) that depends the intensity of the reaction with fractions "a" or "b". In this respect, the fact that fraction "γI" isolated from certain human sera precipitates RNA extracted from TMV and not ribosomal RNA seems to be particularly significant.

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